Clinical concentrations of morphine are cytotoxic on proliferating human fibroblasts in vitro

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Abstract: BACKGROUND: Morphine and other opioids are routinely used systemically and as wound infusions in the postoperative period. Their effect on wound and fracture healing remains unclear. OBJECTIVE: The primary outcome was to assess the potential cytotoxicity of clinically relevant concentrations of morphine on human fibroblasts. DESIGN: Laboratory in-vitro study. SETTING: Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich. MATERIALS: Monolayers of human fibroblasts. INTERVENTION(S): Exposure of human fibroblast monolayers to several concentrations of morphine, for different periods of time, with and without an artificially induced inflammatory process. MAIN OUTCOME MEASURES: Cell count, cell viability, cell proliferation and apoptosis. RESULTS: A concentration, time and exposure-dependent cytotoxic effect of morphine-mediated apoptosis was observed. Simulated inflammatory conditions seemed to lessen toxic effects. CONCLUSION: Cytotoxic effects of morphine are exposure, time and concentration dependent. Simulating aspects of inflammatory conditions seems to increase resistance to morphine cytotoxicity especially in the presence of higher concentration and longer exposure times.

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ORIGINAL ARTICLE

Clinical concentrations of morphine are cytotoxic on proliferating human fibroblasts *in vitro*

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BACKGROUND Morphine and other opioids are routinely used systemically and as wound infusions in the postoperative period. Their effect on wound and fracture healing remains unclear.

OBJECTIVE The primary outcome was to assess the potential cytotoxicity of clinically relevant concentrations of morphine on human fibroblasts.

DESIGN Laboratory in-vitro study.

SETTING Institute of Physiology, Zurich Center for Integrative Human Physiology, University of Zurich.

MATERIALS Monolayers of human fibroblasts.

INTERVENTION(S) Exposure of human fibroblast monolayers to several concentrations of morphine, for different periods of time, with and without an artificially induced inflammatory process.

MAIN OUTCOME MEASURES Cell count, cell viability, cell proliferation and apoptosis.

RESULTS A concentration, time and exposure-dependent cytotoxic effect of morphine-mediated apoptosis was observed. Simulated inflammatory conditions seemed to lessen toxic effects.

CONCLUSION Cytotoxic effects of morphine are exposure, time and concentration dependent. Simulating aspects of inflammatory conditions seems to increase resistance to morphine cytotoxicity especially in the presence of higher concentration and longer exposure times.

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Introduction

As opioids are still the cornerstone drugs for treatment of severe pain,[1] morphine plays a key role in postoperative pain management. However, opioid use may be restricted because of side-effects such as respiratory depression, nausea, constipation, addiction and tolerance. In the late 1980s, several studies showed that not only do opioids activate receptors in the brain and spinal cord but also activate receptors on peripheral sensory neurons.[1,2] Since then, different opioid pathways outside the central nervous system have been described, and new techniques such as intra-articular injections (local infiltration analgesia) and topical wound application have been introduced into clinical practice.[3–5]

Many studies have focussed on NSAIDs as potential causes for non-union after fracture repair,[6] but, in a retrospective study, systemic opioids were also implicated in these complications.[7] However, this latter finding has not been reproduced in a prospective study. The mechanism for toxicity after systemic opioids remains unclear, but in investigations of animal models where different mechanisms of cellular regulation have been the focus of attention and in various clinical trials, direct cell toxicity following topical application of opioids has been implicated as a possible explanation. However, in contrast to such observations, in an in-vitro setting, Haasters *et al.*[8] observed no effect of morphine on either cell survival, cell metabolism or apoptosis in human hamstring-derived stem/progenitor cells. Similarly, studying human osteoblasts in an in-vitro environment, Matziolis *et al.*[9] observed no negative effects of tramadol, at clinically used concentrations. Owing to the development of modern postoperative pain therapy models (wound infiltration, continuous wound infusion) and the crucial role of fibroblasts in wound healing, we designed this in-vitro study to assess possible morphine

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cytotoxicity and its correlation with drug concentration and exposure times of up to 9 days. Apoptotic pathways were primarily investigated as possible mechanisms. In addition, the effects of simulated aspects of inflammation on cytotoxicity were also assessed. Despite the negative findings in the above-mentioned laboratory experiments,[8,9] based on previous clinical results,[7] we expected to observe a moderate cytotoxic effect of morphine on human fibroblasts.

Materials and methods

The in-vitro study was based on our previous protocol involving toxicity of local anaesthetics on human fibroblasts.[10]

Cell cultures

Human osteosarcoma cells (LGC Standard GmbH, Wesel, Germany), which are osteoblast-like cell types with the morphology of human fibroblasts (especially proliferative wound fibroblasts[11]) were cultured in α-modified Eagle's medium (LGC Standard GmbH, Wesel, Germany) with 10% foetal bovine serum (LGC Standard GmbH, Wesel, Germany) and 10 000 IU l^{-1} penicillin/streptomycin (LGC Standard GmbH, Wesel, Germany) at 37°C and 5% CO_2.

Drugs

Preservative free morphine (Morphin-HCL, Sintetica SA, Mendrisio, Switzerland) was obtained from our hospital pharmacy. Serial dilutions were performed to achieve concentrations ranging from 71.3 to 570.68 μg ml^{-1}, corresponding to plasma concentrations measured in clinical practice.[12,13]

Peak plasma morphine concentrations are about 0.001 71 μg ml^{-1} mg^{-1} morphine after immediate release morphine (time to peak, 1 h), 0.000 85 μg ml^{-1} mg^{-1} morphine for controlled release morphine (time to peak, 2.7 h) and 0.000 1425 μg ml^{-1} mg^{-1} morphine for once-daily morphine (time to peak, 8.5 h). The range chosen in this study should simulate different concentrations after injection of morphine (1000 μg ml^{-1}) into a joint/tissue.

Experimental groups

In group 1, cells were exposed to morphine for 2 days, followed by incubation with normal growth medium without morphine for a further 7 days. In group 2, cells were continuously exposed to morphine for the full 9 days. Control cells were incubated with growth medium throughout. In all groups, the medium, with the appropriate concentration of morphine, was changed every second day. Tests were performed on days 3, 6 and 9.

To assess the effect of inflammation, three further groups were studied. These groups corresponded to the above three groups with the addition of 20 ng ml^{-1} lipopolysaccharide (LPS; Fluka, Buchs, Switzerland) added to the culture medium throughout 9 days.

Cell count

Cells were counted using fluorescence DNA quantitation assay (Sigma-Aldrich, Buchs, Switzerland) as described before.[14] Briefly, Hoechst 33258 (bisBenzimide H 33258, Sigma-Aldrich, Buchs , Switzerland) binds specifically to adjacent adenine–thymine base pairs of DNA. The resulting fluorescence changes can be measured efficiently at an excitation wavelength of 360 nm and an emission wavelength of 465 nm.

Cell viability

Tetrazolium bromide assay (Sigma-Aldrich, Buchs, Switzerland) was used as described before.[15] Briefly, in living cells, mitochondrial dehydrogenases catalyse tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] into formazan crystals. This reaction can be quantified by ELISA (wavelength 570 to 620 nm).

Cell proliferation

Colorimetric bromodeoxyuridine (BrdU; Invitrogen Ltd, Paisley, Scotland, UK) analysis was used as described before.[16,17] Briefly, during cell division, BrdU (an analogue of thymidine) is incorporated into new DNA. BrdU incorporation can be quantified with an ELISA reader at 450 nm (reference wavelength 620 nm).

Apoptosis

Caspase-3 activity was determined by measuring proteolytic cleavage of the fluorogenic caspase-3 substrate, Ac-Asp-Glu-Val-Asp-AMC (Calbiochem, Laeufelfingen, Switzerland), as described before.[10] Briefly, cells were washed once and incubated with lysis buffer containing 0.1% Triton-100, Sigma-Aldrich, Buchs , Switzerland. The subsequent fluorescence was measured at an excitation wavelength of 360 nm and an emission wavelength of 465 nm. Camptothecin was used as a positive control (4 μmol l^{-1}).

Twelve samples for each of the above individual tests at each time point were split evenly between two separate 12 × 8 Nunc MaxiSorp flat-bottom 96-well plate (Sigma-Aldrich, Buchs, Switzerland) for analysis, and the results averaged.

Statistical analysis

Values are expressed as mean (SD). Results are presented as a percentage of control. Cell count, viability, proliferation rate, caspase-3 activity and results after LPS exposure were analysed using three-way analysis of variance. Spearman's rank correlation coefficient was computed to assess the relationship between the different variables. OriginPro 8G (OriginLab, Northampton, Massachusetts, USA) and SPSS (SPSS Vers. 17.0, Inc.,

Table 1 Comparison between 2 days' (group 1) and continuous (group 2) exposure to morphine 570.68 μg ml^{-1}

	DNA		MTT		BrdU		Caspase-3	
	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2	Group 1	Group 2
Day 3	71.2 (8.60)	50.9 (1.78)	92.5 (2.68)	107.8 (8.22)	145.6 (4.06)	131.9 (5.14)	19.2 (11.01)	1288.1 (50.79)
Day 6	80.2 (4.11)	22.0 (5.05)	99.2 (4.20)	137.6 (5.06)	149.8 (10.15)	153.4 (6.05)	99.6 (11.15)	322.1 (21.01)
Day 9	52.1 (7.08)	0 (0)	56.0 (3.49)	0 (0)	140.0 (8.31)	0 (0)	14.0 (7.40)	0 (0)

Results are expressed as a percentage of control, mean (SD). BrdU, bromodeoxyuridine; MTT, tetrazolium bromide.

Chicago, Illinois, USA) were used for statistical analyses. A probability of $P < 0.05$ was considered statistically significant.

Results

Cell numbers (DNA quantitation)

Cell count was significantly lower in the presence of morphine when compared with control ($P < 0.05$). Exposing cells to morphine for 9 days (group 2) led to a significant decrease in cell count when compared with cells with only 2 days' exposure (group 1, $P < 0.001$; Table 1). Simulating aspects of inflammation led to a significantly higher cell count at the higher morphine concentration when compared with cells exposed under normal conditions ($P < 0.001$; Fig. 1a).

After 2 days' exposure to the highest concentration of morphine, simulating inflammatory conditions led to a significantly higher cell count after 9 days when compared with cells exposed without such simulation ($P < 0.001$; Fig. 1b). At the highest morphine concentration, no cells survived 9 days of continuous exposure, irrespective of simulated inflammation (Fig. 1a).

Cell viability (tetrazolium bromide activity)

Cell viability in surviving cells was significantly lower after exposure to morphine for 2 days (group 1) when compared with cells exposed 3 and 6 days (group 2). No cell survived 9 days of continuous exposure at high concentrations (Table 1). Simulating aspects of inflammatory conditions led to a significantly higher cell viability when compared with cells exposed under normal conditions ($P < 0.001$; Fig. 2).

Cell proliferation (bromodeoxyuridine activity)

Proliferation rate was significantly higher in surviving cells after exposure to morphine ($P < 0.001$; Table 1). In cells exposed for 2 days (group 1) to high concentration of morphine, simulating aspects of inflammatory conditions led to a significantly lower proliferation rate when compared with cells exposed to morphine without such simulation (Fig. 3).

Apoptotic pathway (caspase-3 activity)

Limiting exposure to high concentrations of morphine to 2 days (group 1) led to significantly lower caspase-3 activity when compared with the 9 days' exposure (group 2, $P < 0.001$; Table 1). Simulating aspects of inflammatory conditions led to a significantly reduced caspase-3 activity compared with cell without such simulation ($P < 0.001$; Fig. 4).

Discussion

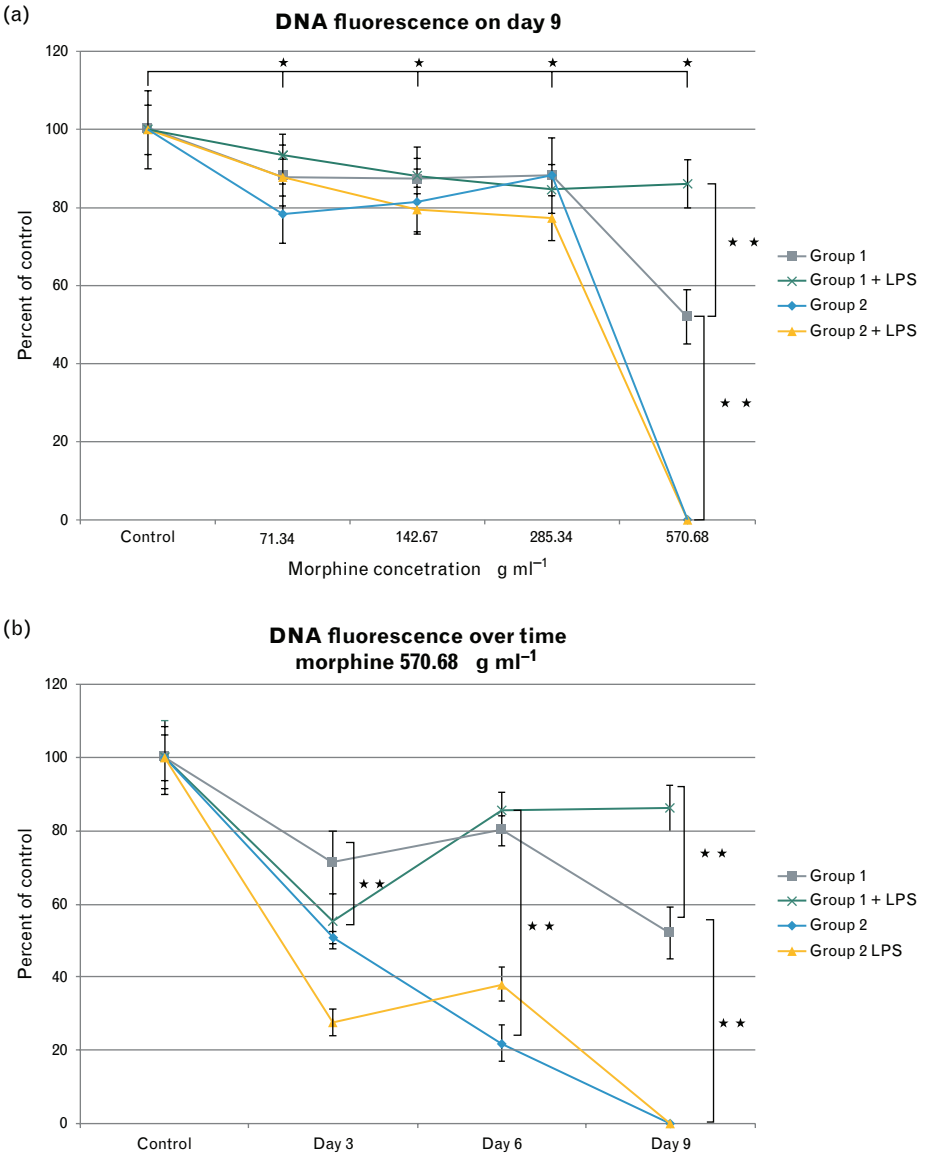
The in-vitro study shows that limiting exposure of fibroblasts to lower concentrations of morphine and limiting exposure to 2 days reduces its cytotoxicity when compared with high concentrations and longer exposure times (9 days). It also shows that exposing cells to morphine in an environment simulating aspects of inflammation leads to a higher cell count, higher cell viability and lower activation of the apoptotic pathway when compared with a non-inflammatory state.

The present study protocol was based on a previous study involving toxicity of local anaesthetics on human fibroblasts.[10] The influence played by opioids on fibroblasts and osteoblasts remains unclear. In a retrospective study of humeral shaft fractures, Bhattacharyya *et al.*[7] associated systemic opioid intake with non-union after 61 to 90 days. In contrast, in-vitro studies of opioids did not show toxic effects on cell viability of osteoblasts[9] or human hamstring-derived stem/progenitor cells.[8] As postoperative local application of morphine for pain therapy is becoming more common,[4,5] we decided to investigate its possible cytotoxicity.

We chose a limited (2 days, group 1) and a continuous exposure (9 days, group 2) to simulate single shot and continuous morphine usage, respectively. Although, in clinical practice, continuous exposure is seldom longer than 2 days, we decided to include a 9 days' follow-up as no clinical study has shown for how long and at what concentrations morphine remains in the tissues after either a single injection or a continuous infusion.

Furthermore, by simulating inflammation with the addition of LPS to the cell cultures in our current study, we also attempted to observe the effect of the inflammatory process as it might be found in the acute postoperative phase. The effects induced by systemic administration of LPS have been described in the literature,[18] and – recently – in an in-vivo mouse model, the topical application of LPS at the wound surface has been observed to influence the inflammatory process and so promote wound healing on the injured skin.[19] However,

Fig. 1

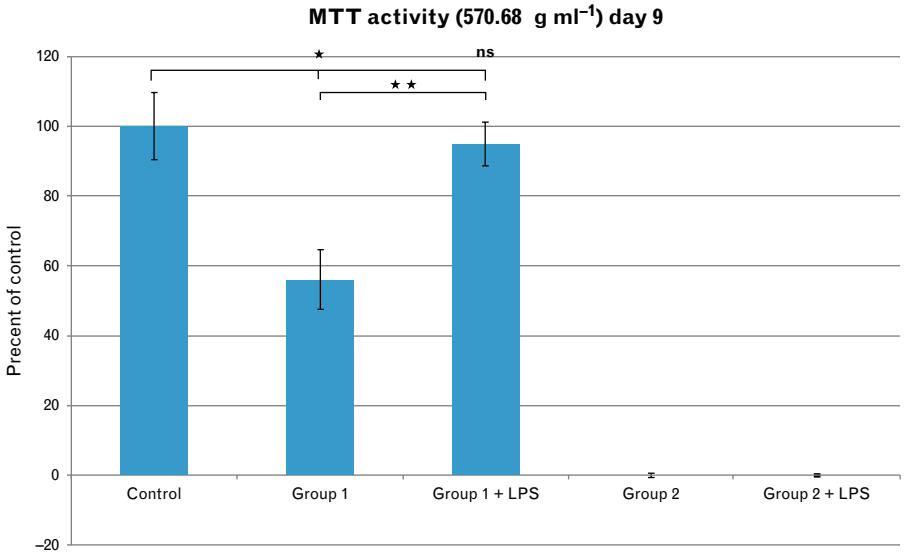


(a) Cell numbers (DNA quantification): dosis effects. Results are a percentage of control values. Cell count was significantly lower in the presence of morphine when compared with control ($P < 0.05$). At 570.68 μg ml^{-1} concentration of morphine, simulating inflammatory conditions with LPS led to a significantly higher cell count when compared with cells exposed under normal conditions without LPS ($P < 0.001$). *$P < 0.05$, **$P < 0.001$. (b) Cell numbers (DNA quantitation): time course. In group 1 (2 days' exposure to morphine), simulating inflammatory conditions with LPS led to a significantly higher cell count at 9 days when compared with cells exposed under normal conditions without LPS ($P < 0.001$). When cells were exposed continuously to morphine, simulated inflammation with LPS had no effect. **$P < 0.001$. LPS, lipopolysaccharide.

LPS administration is not validated in an in-vitro model of wound inflammation.

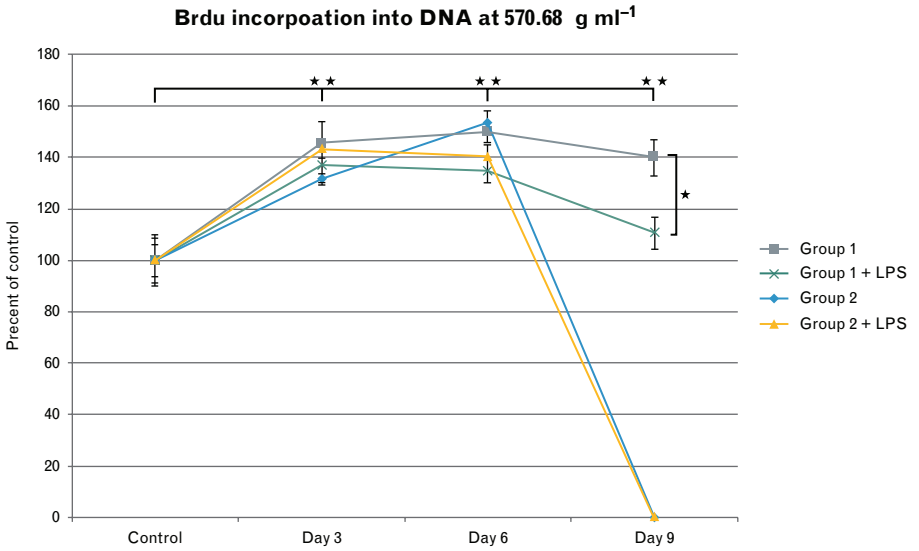
Our results show a small reduction in cell numbers with low/moderate morphine doses and a significant reduction in cell numbers in the presence of the highest morphine concentration, suggesting the possibility of a 'toxic threshold' between morphine doses of 285.34 and 570.68 μg ml^{-1} (Fig. 1a). Furthermore, limiting morphine exposure to 2 days allowed cells to survive 9 days, whereas no cells survived after 9 days of continuous exposure to high morphine concentrations (Fig. 1a and b). Cell viability in the surviving cells was also compromised after 9 days in group 1 (Fig. 2). These findings concur with results from different animal experiments which describe a time-dependent relationship between topical morphine treatment and wound healing, with a reduction of myofibroblasts, the progenitors of fibrocytes, which leads to reduced scar formation.[20,21] This is in accordance with the work of Lam *et al.*[22] in mice; they reported that

Fig. 2



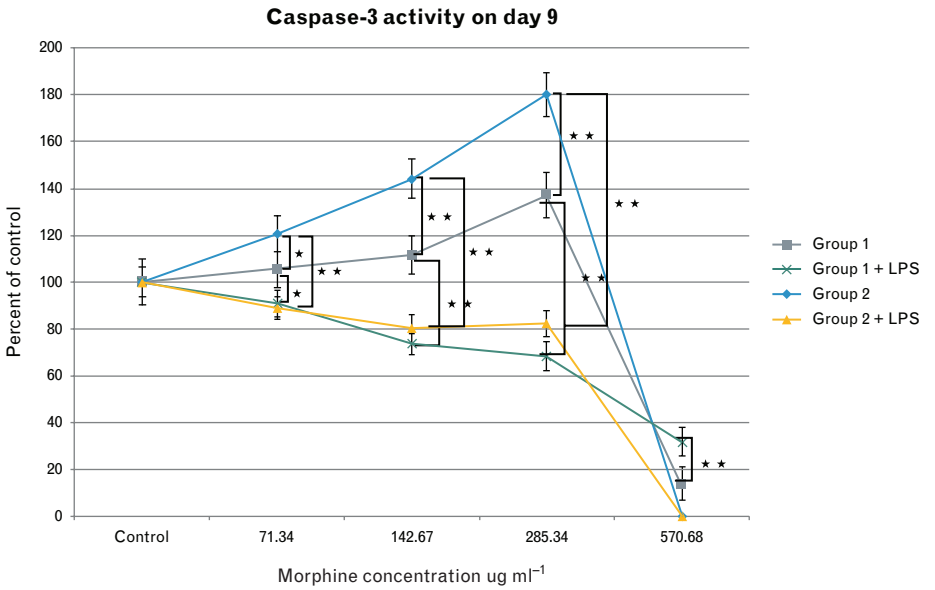
Cell viability (tetrazolium bromide activity). At the highest concentration of morphine, no cells survived 9 days of continuous exposure to morphine, with or without simulated inflammation. In the cells exposed to morphine for 2 days (transient exposure), at this high concentration of morphine (group 1), compared with the control cells, there was a significantly lower viability in the surviving cells ($P < 0.05$). Viability of these transiently exposed cells was significantly higher when inflammatory conditions were simulated (group 1 + lipopolysaccharide, $P < 0.001$). $^{*}P < 0.05$, $^{**}P < 0.001$. NS, not significant.

Fig. 3



Cell proliferation (BrdU activity). Proliferation rate was significantly higher after exposure to morphine ($P < 0.001$). In cells transiently exposed (group 1) to high concentration of morphine, simulating aspects of inflammatory conditions lead to a significantly lower proliferation rate when compared with cells exposed to morphine under normal conditions. Cell proliferation (BrdU activity). At 3 and 6 days, the cell proliferation rate after exposure to morphine (570.68 μg ml^{-1}) was significantly higher than the control cells ($P < 0.001$). In group 1, cells transiently exposed to this morphine concentration, simulating inflammatory conditions with LPS resulted in a significantly lower proliferation rate when compared with cells exposed to morphine under normal conditions without LPS ($P < 0.05$). At this concentration, practically no cell survived 9 days of continuous exposure to morphine. $^{*}P < 0.05$, $^{*}P < 0.001$. BrdU, bromodeoxyuridine; LPS, lipopolysaccharide.

Fig. 4



Apoptotic pathway (caspase-3 activity). When compared with cells only transiently exposed to the drug (group 1), continuous exposure to morphine (group 2) resulted in a significantly increased caspase-3 activity. Simulating inflammatory conditions with LPS (group 2 + LPS) resulted in significantly less caspase-3 activity when compared with cells exposed under normal conditions without LPS ($P < 0.001$). At the highest concentration of morphine (570.68 $\mu g\,ml^{-1}$), no cells survived 9 days of continuous exposure. $^{*}P < 0.05$, $^{**}P < 0.001$. LPS, lipopolysaccharide.

high-dose morphine (intraperitoneal injection of 20 $mg\,kg^{-1}\,day^{-1}$ for 14 days) resulted in excessive generation of superoxide anions and impairment of angiogenesis with consequent delay in wound healing. A single injection of morphine 20 $mg\,kg^{-1}$ is equivalent to a plasma concentration of 2.25 $\mu g\,ml^{-1}$.[23] The plasma morphine levels after 14 days of continuous intraperitoneal administration in that investigation are unknown, as Lam *et al.*[22] did not measure them. But these results do show that a negative impact on cell viability can be used for a positive clinical outcome. Our results are in contrast to those of Haasters *et al.*,[8] who showed only a small reduction in the metabolic activity of human tendon stem/progenitor cells and no cell death after a 6-h exposure to a morphine concentration of 250 $\mu g\,ml^{-1}$, which is within the range of our concentrations (71.3 to 570.68 $\mu g\,ml^{-1}$). However, this difference in findings between their results[8] and our present study could be explained by the very different exposure times (6 h[8] versus 2 and 9 days in our present study) and the moderate concentration chosen.[8] In rats, Hanci *et al.*[24] observed no evidence of histological or ultrastructural damage to wound tissue 8 days after a single infiltration with 3 ml of 5% tramadol. These results, contrasting with our findings, could also be explained by the shorter opioid exposure time.

In our study, until all the cells died at the highest morphine concentration, the cell proliferation rate was significantly higher after exposure to morphine (Fig. 3). These findings could be explained by modulation of early growth-related genes as *c-jun*, *c-fos* and *c-myc* in kidney fibroblasts,[25] despite the fact that higher concentrations of morphine suppress fibroblast proliferation.[25,26] The significance of these finding needs further investigation.

Caspase-3 activity was significantly higher after exposure to morphine in a concentration and exposure-dependent fashion. Increasing the exposure time up to 9 days (group 2) led to significantly higher caspase-3 activity (Fig. 4) in the surviving cells, hence predicting the decline in cell count with time (Fig. 1b). These findings are in accordance with the results of Hatsukary *et al.*[27] and Takeuchi *et al.*[28] who showed a dose-dependent activation of caspase-3, but are in contrast with Haasters *et al.*[8] who found no induction of apoptosis after a 6-h exposure to a morphine concentration of 250 $\mu g\,ml^{-1}$. Again, differences in exposure time could explain these contrasting outcomes.

Simulating aspects of inflammation led to a significantly higher cell count after 2 days' exposure to the highest morphine concentration (570.68 $\mu g\,ml^{-1}$; Fig. 1a): cell count increased 4 days after withdrawal of morphine and remained constant thereafter in contrast to cells cultured under normal conditions (Fig. 1b). Cell viability was also higher (Fig. 2). Both the cell proliferation rate (Fig. 3) and caspase-3 activity (Fig. 4) were lower in cells

exposed to simulated aspects of inflammation. The reduction in activation of the apoptotic cell death pathway might explain the higher cell counts in cells exposed to aspects of inflammation and might be interpreted as a protective mechanism from morphine cytotoxicity. There are no published in-vitro data with which to compare our results.

These findings could also indicate that experiments conducted with cells harvested from patients during or after surgery, who are already exposed to an inflammatory stressor,[9,29] might confound toxicity results. However, the impact of inflammation on morphine-induced cellular toxicity needs further investigation.

This study has some limitations. First, we did not include a second opioid to assess a potential drug-specific impact on cell viability. Tramadol is also currently used for wound infiltration[30] and would have been an appropriate candidate despite negative results in a previous study.[9] We focused on morphine, the most widely used opioid. Second, we did not explore other mechanisms of cell death apart from apoptosis. Nevertheless, apoptosis is one of the main mechanisms of cell death after exposure to NSAIDs and local anaesthetics.[10,31,32] Third, we did not use recently cultured cells from human tissue but used cells from an existing cell line. This model is well established and has already been used by our group to study the toxic effects of local anaesthetics.[10] Fourth, we investigated cytotoxicity only at three time points (3, 6 and 9 days) in our two groups, and increasing the number of time points investigated may have allowed us to make a more general recommendation on limiting exposure times to morphine in clinical settings. Fifth, as previously mentioned, in clinical practice, local tissue exposure to 9 days of morphine is not usual, longer morphine use is generally by systemic administration. However, no clinical study has shown how long and at which concentrations morphine remains in the tissues either after single or continuous topical use, or after systemic administration.

In conclusion, according to the model used, limiting tissue exposure to morphine to shorter periods of time and to lower concentrations reduces morphine toxicity on fibroblasts. In addition, the presence of an inflammatory reaction in the tissues after surgery could provide some protection from the toxic effects of morphine. How our in-vitro results might impact on the clinical use of morphine and other opioids for intra-articular or wound application must await further evaluation.

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Conflicts of interest: none.

Presentation: none.

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